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PRINCIPAL INVESTIGATOR: Judith Campisi, Ph.D.

CONTRACTING ORGANIZATION: Buck Institute for Age Research  
Novato, California 94945-1400

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<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Buck Institute for Age Research Novato, California 94945-1400  E-Mail: <a href="mailto:jcampisi@buckinstitute.org">jcampisi@buckinstitute.org</a>			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
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<b>13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)</b>  Aging is the single largest risk factor for developing breast cancer and is thought to be due the convergence of the accumulation of mutations together with the accumulation of senescent cells. Our working hypothesis is that senescent epithelial cells can cause preneoplastic or neoplastic changes in its neighbors, and that these changes will be manifest when cells are cultured in three dimensions, which more closely mimics the natural tissue environment than conventional two dimensional cultures. To test this hypothesis, we have successfully established two and three dimensional culture models of normal human mammary epithelial cells (HMECs) with and without a functional 16 tumor suppressor pathway. We have also created preneoplastic HMECs by introducing defined genes with oncogenic potential, particularly genes that selectively inactivate the p53 or pRB tumor suppressor pathways. We have begun to use these, and frankly neoplastic human mammary epithelial cells, in the two and three dimensional culture assays.				
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## INTRODUCTION

Aging is the single largest risk factor for developing breast cancer and is thought to be due the convergence of the accumulation of mutations together with the accumulation of senescent cells. Our working hypothesis is that senescent epithelial cells can influence its neighbors, and that these changes will confer preneoplastic or neoplastic phenotypes on the neighboring cells. Moreover, we hypothesize that these changes will be most clearly manifest when cells are cultured in three dimensions, which more closely mimics the natural tissue environment than conventional two dimensional cell culture systems. To test this hypothesis, we proposed to establish two and three dimensional culture models of normal human mammary epithelial cells (HMECs) with and without a functional 16 tumor suppressor pathway. We also proposed to create and incorporate into these culture models preneoplastic HMECs that lack functional p53 and/or pRB tumor suppressor pathways, and frankly neoplastic human mammary epithelial cells. We proposed to monitor the cell culture models for indices of cell proliferation and differentiation, particularly morphological differentiation (ability to form structures present in normal or neoplastic breast tissue).

## BODY

1. Establishment of two dimensional cell culture models of presenescent and senescent normal human mammary epithelial cells (HMECs) that retain or lack a functional p16 tumor suppressor pathway (part of approved statement of work #1).

a) We have obtained and optimized culture conditions for HMECs. We optimized culture conditions for both preselected (p16+, p16 tumor suppressor pathway intact) and postselected (p16-, p16 pathway inactivated due to spontaneous methylation of the p16 promoter) HMECs. We have cultured both types of HMECs until they have undergone replicative senescence.

Research and findings: We cultured HMECs (strain 184) in mammary epithelial growth medium (MEGM), passaging continually for several weeks until there was no increase in cell number over a 2 week interval and the percentage of cells that incorporated bromo-deoxyuridine (BrdU), indicative of DNA synthesis, was <10% over a three day interval (labeling index) (replicative senescence). In two independent cultures, preselected HMECs underwent 8-12 population doublings (PDs) before cell growth ceased and the labeling index declined to 3-5%. This relatively short replicative life span of preselected HMECs makes it difficult to perform experiments with them, owing primarily to the rapid loss of presenescent cells from the culture. In an attempt to extend the replicative life span of preselected HMECs, we also cultured them in a reduced oxygen atmosphere (3% oxygen, which more closely approaches physiological oxygen levels). Reduced oxygen made little or no difference in the number of PDs achieved before the cells arrested growth, indicating that the limited

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replicative life span of these cells is not due to the stress of culture in atmospheric oxygen levels. Postselected HMECs, by contrast, underwent 20-30 PDs before cell growth ceased and the labeling index declined to 8-9%. These cells therefore have a replicative life span that is adequate for many presenescent-senescent comparisons. In both types of cultures (pre- and post-selected), upon replicative senescence, 75-80% of the cells expressed the senescence marker SA-Bgal.

We have also explored the use of X-irradiation to induce rapid senescence of HMECs, which will allow us to study the phenotypic consequences of senescent HMECs much more efficiently than the long culture times required for replicative senescence. The phenotypes of replicatively senescent and irradiation-induced senescent HMECs were indistinguishable with respect to cell proliferation, cell morphology and expression of the senescence marker, senescence-associated beta-galactosidase (SA-Bgal).

Research and findings: Although 5 Gy X-ray are generally sufficient to induce senescence in >95% of human fibroblasts, we found that postselected HMECs were incompletely (<50%) arrested by this radiation dose. We therefore used increasingly higher X-ray doses until the cells arrested growth with little or no cell death. At 10 Gy X-ray, most (>95%) of the cells in the culture arrested growth as determined by achieving a labeling index of <10% within 2-3 days after irradiation. In addition, over an additional 3-5 days, they developed the typical enlarged, flattened senescent morphology. Between 75 and 80% of these irradiated cells stained positive for SA-Bgal.

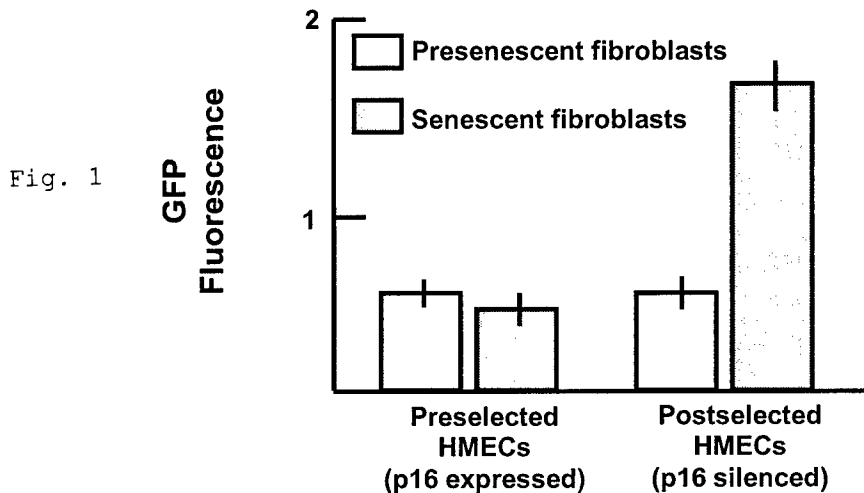
b) We have obtained and optimized culture conditions for mammary fibroblasts. We have cultured the cells to replicative senescence, and have used X-irradiation to induce rapid senescence. As we found with HMECs, X-irradiated fibroblasts were phenotypically identical to replicatively senescent fibroblasts by the criteria of cell proliferation, morphology and SA-Bgal expression.

Research and findings: We used strain 184 human mammary fibroblasts, obtained from the same donor as the pre- and post-selected HMECs. The mammary fibroblasts grew well under culture conditions optimized for other fibroblast strains (Dulbecco's modified Eagle's medium, 10% fetal calf serum, 4 mM glutamine). They underwent 25-30 PDs before reaching replicative senescence, as determined by cessation of growth with a labeling index of <10%, development of an enlarged flattened morphology, and >75% of the cells staining positive for SA-Bgal. Also similar to other fibroblast strains, human mammary fibroblasts responded to irradiation with 5 Gy X-ray by undergoing a senescence arrest within 2-3 days, showing the typical senescent morphology, low (<10%) labeling index, and high (>75%) SA-Bgal staining.

c) We have established two dimensional co-cultures models in which we have mixed presenescent p16+ or p16- HMECs with presenescent or senescent fibroblasts. We found that p16+ HMECs were unaffected by the presence of senescent fibroblasts in two dimensions. By contrast, p16- HMECs were stimulated to proliferate two- to three-fold by the presence of senescent fibroblasts. This finding suggests that the p16 tumor suppressor pathway (or the pRB pathway, which is positively regulated by p16), is responsible for conferring sensitivity to a senescent stromal microenvironment on HMECs. It also suggests that loss of p16 expression (by methylation or mutation) *in vivo* may be an important risk factor for the development of age-related breast cancers.

Research and findings: We expressed the green fluorescent protein (GFP) in pre- and post-selected HMECs using a retroviral construct we created and high titer retroviral preparations. This manipulation allowed us to quantify HMEC proliferation in mixed co-cultures with fibroblasts using quantitative fluorescence imaging. We then prepared lawns of presenescent and senescent fibroblasts on two dimensional culture dishes, replaced the medium with serum-free medium, and seeded onto the fibroblast lawns  $1 \times 10^4$  GFP-expressing HMECs, either preselected (p16 unmethylated and therefore expressed) or postselected (p16 methylated and

therefore not expressed). After five days of co-culture, we quantified the amount of HMEC proliferation by measuring GFP fluorescence. The results are shown below. HMECs, whether pre- or post-selected, proliferated similarly on presenescent fibroblast lawns (white bars). On senescent lawns (gray bars), however, postselected HMECs were selectively stimulated to proliferate. Vertical bars are standard deviations.



d) We are currently determining the effect of senescent fibroblasts in two dimensional co-culture with p16+ and p16- HMECs on differentiation characteristics of the HMECs. Our preliminary results suggest that p16- HMECs are susceptible to losing proper subcellular localization of the cell adhesion protein E-cadherin in the presence of senescent but not presenescent stromal fibroblasts. This result is still preliminary. It needs to be confirmed and supported by investigation of additional differentiation markers, but may suggest that a senescent stromal microenvironment may promote loss of optimal epithelial cell-cell communication in the aged breast.

**Research and findings:** We immunostained co-cultures of HMECs on presenescent and senescent fibroblast lawns with an anti-E-cadherin antibody, followed by staining with a fluorescent secondary antibody. E-cadherin immunostaining is generally most prominent at sites of cell-cell junctions. In the co-cultures, E-cadherin staining was less intense at cell-cell contact regions when postselected HMECs were cultured on lawns of senescent fibroblasts compared to all other conditions (postselected HMECs on presenescent fibroblast lawns, and preselected HMECs on both presenescent and senescent fibroblast lawns).

e) We are currently optimizing conditions to co-culture senescent and presenescent HMECs in two dimensions in order to determine the phenotypic consequences of the senescence of the epithelial cells on their epithelial neighbors.

**Research and findings:** We described in section 1a above the culture of HMECs to replicative senescence and the rapid induction of senescence using X-irradiation. In order to perform co-culture experiments with these epithelial cells, we must incubate them in medium containing no or minimal growth factors so that any growth stimulation we see can be attributed to the cells, not to exogenously supplied growth factors. Our initial experiments used postselected HMECs because their longer replicative life span made them easier to manipulate and because our experiments with HMECs co-cultured with fibroblasts showed that postselected HMECs were selectively sensitive to the senescent fibroblast environment. We found that neither presenescent nor senescent postselected HMECs thrived in growth factor-free medium, as determined by the

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gradual appearance of a vacuolated cytoplasm and gradual loss of cell adherence to the culture dish. We found that supplementing the medium with low levels (10 ng/ml) of insulin helped maintain viability, although the cells did not thrive as well as in complete medium with growth factors.

2. Initiate marker characterization of senescent cells using high-throughput assays of secreted factors expressed by senescent fibroblasts and HMECs (part of approved statement of work #1).

a) As proposed, high-throughput assays of expressed genes can give a much broader picture of the senescent phenotype. One such assay is the use of high-density cDNA microarrays; another, which more specifically probes the secretory phenotypes of cells, is the use of high-density antibody arrays. We chose to begin characterization using antibody arrays because our preliminary experiments indicated that these arrays have lower false positive rates, and are more pertinent to the phenotypic change we hypothesize is crucial for the effects of senescent cells on the tissue microenvironment and neighboring cells. We have optimized conditions to use these arrays with chemiluminescent detection, and in a small pilot experiment have validated their use by RT-PCR and western blot analysis of conditioned medium from senescent cells.

**Research and findings:** The human arrays with which we initiated this study are commercially available (Raybiotech) and contain 120 antibodies directed against different human cytokines. Our initial efforts to use this technology entailed several optimization experiments for which used human mammary fibroblasts induced to senesce by X-irradiation. The results of these optimization experiments allowed us to determine 1) the number of cells and medium volumes needed to detect signals from conditioned media; 2) culture regimens to allow presenescent cells to enter a quiescent state prior to collection of conditioned medium (in order to make valid comparisons with conditioned medium from postmitotic senescent cells); 3) the need to concentrate the conditioned media 3-4 fold, and the best method to do so without detectable loss of soluble factors; 4) the intervals needed for incubation of conditioned media with the arrays, and incubations needed for chemiluminescent detection. An example of an enlarged portion of an optimized array and the preliminary result it provides is shown below. Signals outlined in blue are positive and negative controls; signals circled in red are soluble factors detected by the array, identified on the right.

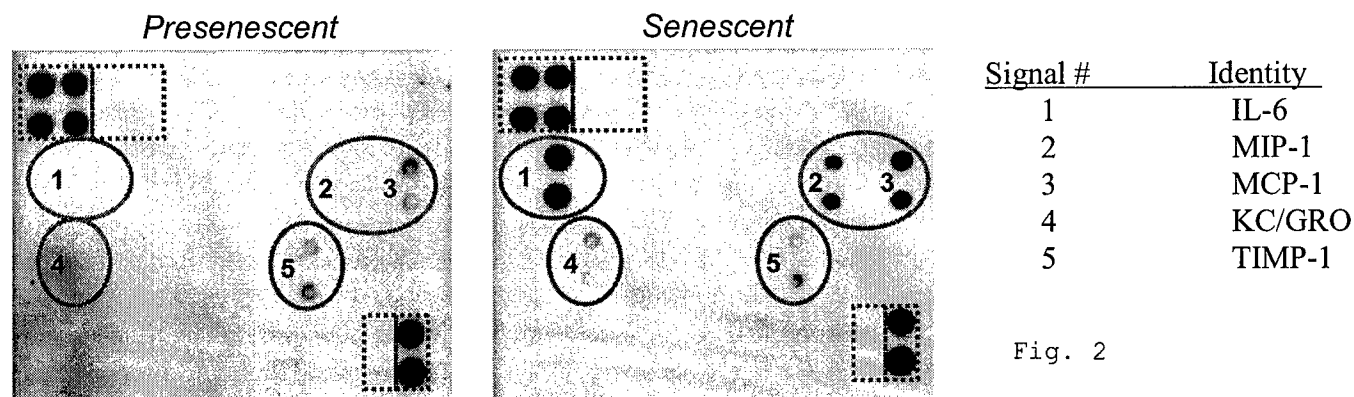


Fig. 2

The results indicate little or no change in TIMP-1 secretion (independently confirmed by RT-PCR) between presenescent and senescent cells, but significantly increased secretion of IL-6 (confirmed), MIP-1, MCP-1 and KC/GRO by senescent cells. Because the signal intensities vary so much, chemiluminescent detection is not strictly quantitative. Nonetheless, the preliminary and semi-quantitative conclusion is that senescent cells secrete elevated levels of several cytokines that have inflammatory and/or cell migratory activities.

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3. Initiate establishment of three dimensional culture and co-culture model systems to evaluate effects of senescent cells on HMECs under more physiological culture conditions (part of approved statement of work #2).

a) To begin to explore the role of senescent cells on HMECs under more physiological cell culture conditions, we have begun to establish three dimensional culture models. First, we have optimized conditions for alveolar morphological differentiation of HMECs, using a commercially available preparation of basement membrane components. This model is the most standard model used for studying HMEC differentiation. We have shown that both p16+ and p16- HMECs form well-organized alveolar structures in this model, and are beginning to explore the effects of incorporated senescent HMECs. We have also optimized conditions for ductal differentiation of HMECs, in which the HMECs are cultured in three dimensions in collagen into which fibroblasts have been incorporated. Our preliminary data suggest that the presence of senescent fibroblasts disrupt both alveolar and ductal HMEC morphogenesis. These findings suggest that a senescent stromal microenvironment may alter the morphological differentiation of HMECs in human breast tissue during aging.

Research and findings: We used strain 184 HMECs, MEGM and Matrigel applied in a drip assay previously optimized for mouse mammary epithelial cells and found that pre- and post-selected HMECs formed well-ordered alveolar-like structures within 7-10 days (morphological differentiation). We used the line MCF10A, which behaves identically to strain 184 cells in the Matrigel drip assay, to begin to explore the influence of a senescent stromal microenvironment on HMEC morphological differentiation. An example of the well-ordered alveolar structures formed in the presence of presenescent stromal fibroblasts, and the relatively disorganized structures formed in the presence of senescent stromal fibroblasts is show below.

PRESENECENT

SENECENT

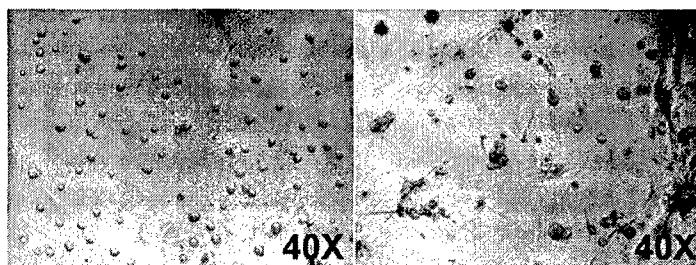


Fig. 3

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These experiments were carried out by myself (10% effort), the part time effort of a research technician (50% effort) and a postdoctoral scientist, currently working at 100%. The postdoctoral scientist will continue to work at 100% for the next few months in order to accelerate characterization of the HMEC phenotypes that are influenced by senescent cells.

#### KEY RESEARCH ACCOMPLISHMENTS

- Determined that the relatively rapid senescence of preselected HMECs is not due to atmospheric oxygen
- Determined that HMECs permanently arrest growth with a senescent phenotype following irradiation with 10 Gy X-rays
- Determined that human mammary fibroblasts arrest growth with a senescent phenotype after 5 Gy X-rays
- Developed conditions for two dimensional co-culture models of HMECs and fibroblasts
- Determined that loss of the p16 pathway (in postselected HMECs) confers sensitivity of HMECs to senescent stromal (fibroblast) environment in two dimensional co-culture assays
- Established conditions to successfully characterize secretory phenotype of senescent cells using antibody arrays.
- Preliminary results from antibody arrays indicate increased secretion of inflammatory and cell migratory cytokines by senescent cells.
- Determined conditions for three dimensional culture models of HMEC morphogenesis.

#### REPORTABLE OUTCOMES

No reportable outcomes thus far.

#### CONCLUSIONS

We have made excellent progress in establishing the proposed two dimensional and three dimensional culture systems of p16+ and p16- HMECs with or without stromal fibroblasts, obtaining senescent cells for incorporation into two and three dimensional co-culture systems, and characterization of proliferative characteristics. We have also made good progress in characterization of senescence markers in order to obtain a more comprehensive understanding of how senescent cells may alter HMEC phenotype and differentiation.